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A region of human BRCA2 containing multiple BRC repeats promotes RAD51-mediated strand exchange

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ABSTRACT

Human BRCA2, a breast and ovarian cancer suppressor, binds to the DNA recombinase RAD51 through eight conserved BRC repeats, motifs of ~30 residues, dispersed across a large region of the protein. BRCA2 is essential for homologous recombination *in vivo*, but isolated BRC repeat peptides can prevent the assembly of RAD51 into active nucleoprotein filaments *in vitro*, suggesting a model in which BRCA2 sequesters RAD51 in undamaged cells, and promotes recombinase function after DNA damage. How BRCA2 might fulfill these dual functions is unclear. We have purified a fragment of human BRCA2 ($BRCA2_{BRC1–8}$) with 1127 residues spanning all 8 BRC repeats but excluding the C-terminal DNA-binding domain ($BRCA2_{CTD}$). $BRCA2_{BRC1–8}$ binds RAD51 nucleoprotein filaments in a ternary complex, indicating it may organize RAD51 on DNA. Human RAD51 is relatively ineffective *in vitro* at strand exchange between homologous DNA molecules unless non-physiological ions like NH_4^+ are present. In an ionic milieu more typical of the mammalian nucleus, $BRCA2_{BRCI–8}$ stimulates RAD51-mediated strand exchange, suggesting it may be an essential co-factor *in vivo*. Thus, the human BRC repeats, embedded within their surronding sequences as an eight-repeat unit, mediate homologous recombination independent of the $BRCA2_{CTD}$ through a previously unrecognized role in control of RAD51 activity.

INTRODUCTION

BRCA2, a tumour suppressor whose inactivation is associated with hereditary breast and ovarian cancer predisposition, is essential for DNA repair in mammalian cells (1–3). BRCA2-deficient cells are defective in the repair of DNA double-strand breaks by error-free homologous recombination (4), allowing error-prone repair processes to create gross chromosomal re-arrangements that may promote carcinogenesis (2). The role of BRCA2 in homologous recombination has been linked to its functions in the regulation of RAD51, a RecA-related recombinase that forms the nucleoprotein filaments on damaged DNA that are crucial to recombinational repair (5,6). BRCA2 binds directly to RAD51 through 6 of the 8 BRC repeats, ~30 amino acid motifs encoded within the central exon 11 region of all known mammalian *BRCA2* genes (7–9). This feature distinguishes mammalian BRCA2 molecules from simpler eukaryal homologues such as Brh2 in *Ustilago maydis*, which contains a single BRC repeat (10).

Available data concerning the nature and consequences of the interaction between human BRCA2 and RAD51 mediated by the BRC repeats raise an important but unresolved question. On the one hand, peptides encoding isolated BRC repeats such as BRC3 when present in excess can prevent the assembly of RAD51 into nucleoprotein filaments on DNA substrates, and can dissolve pre-formed RAD51 complexes (11). A structural basis for this apparently inhibitory activity is suggested by the fact that BRC repeats can bind to RAD51 by mimicking its mode of self-association; thus preventing multimerization and dispersing existing multimers (12). Conversely, however, there is evidence that isolated BRC repeat peptides under certain conditions can bind to the RAD51 nucleoprotein filament, rather than disrupting it

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(13), in keeping with biological evidence that BRCA2 promotes (rather than inhibits) RAD51 foci formation and DNA recombination in cells (2,4,14). These observations have been reconciled in a hypothetical model proposing that BRCA2 sequesters RAD51 in a monomeric form bound to BRC repeats, until activation by DNA damage signals results in the mobilization of BRCA2–RAD51 complexes, and their targeting to sites of DNA repair where BRCA2 may promote recombination (15). Studies on the dynamics of functional GFP–RAD51 fusion proteins in the nuclei of living cells before and after DNA damage offer support for this view (16), although the lack of direct biochemical evidence that human BRCA2 is a recombination mediator has been noted as an important hiatus in current understanding (17).

How can BRCA2–RAD51 complexes delivered to sites of DNA damage operate to promote repair by homologous recombination? Whilst the function of the C-terminal domain of BRCA2 in recombination can be rationalized by its ability to bind DNA and stimulate RAD51-mediated strand exchange *in vitro* through the displacement of the single-strand DNA-binding protein RPA (18), the role of the BRC repeat-containing region is less clear, given the ability of isolated BRC repeat peptides to both disrupt filaments (11,12) and bind to them (13), depending on the experimental conditions. These studies—and indeed, most previous work on the biochemical function of the BRC repeats—have utilized isolated BRC repeat peptides. However, all mammalian BRCA2 molecules contain eight conserved BRC repeats in the exon 11 region (Figure 1A).

Several features of the organization of the exon 11 region, shared across mammalian evolution, point to the fact that isolated BRC repeat peptides may not accurately reflect the biological activities of the eight BRC repeats embedded within the context of surrounding sequences in exon 11. First, although the exon 11 sequence that flanks the BRC repeats is poorly conserved even in close mammalian species, the spacing that it provides between consecutive BRC repeats has been well conserved in evolution (9). Second, there is a considerably higher degree of sequence conservation between the same BRC repeat of different mammalian species than between different BRC repeats of one species (9,19). This raises the possibility that during evolution, diverse selective pressures have been placed on the BRC repeats at different sites in exon 11, with each BRC repeat adopting an idiosyncratic function (5,19). This is supported by evidence of differences in the functional characteristics of BRC3/BRC4 and BRC7 *in vitro* (11,13). However, the practical difficulties of producing either full-length mammalian BRCA2 (>3000 amino acids) or the BRC repeat-containing exon 11 region (>1000 amino acids) in a form suitable for experimentation, have meant that experiments have thus far been devoted to studying the function of isolated BRC repeats rather than the more physiologically relevant collective function of the eight BRC repeats within the context of the exon 11 region.

To address this, we have purified a 1127 amino acid fragment of human BRCA2 that contains all 8 BRC repeats ($BRCA2_{BRC1–8}$). We show here that purified $BRCA2_{BRC1–8}$ forms a ternary complex with RAD51 and DNA, and is capable of promoting RAD51-mediated strand exchange *in vitro*. Thus, despite the apparently inhibitory effects of isolated BRC repeats, within the framework provided by the exon 11 region, BRC repeats function to stimulate DNA recombination. This novel activity provides direct biochemical evidence that BRCA2 functions as a mediator of homologous recombination in mammalian cells.

MATERIALS AND METHODS

Protein expression, purification and peptide synthesis

A region of human *BRCA2* corresponding to a 1127 amino acids fragment between residues 987 and 2113 of the encoded protein was cloned into a pET28a vector (Novagen) and expressed in Rosetta (DE3) competent cells (Novagen). Cells were lysed in 20 mM Tris–HCl, pH 8.0, 300 mM NaCl and 5 mM DTT containing protease inhibitors (Roche); the insoluble pellet was clarified and washed with 20 mM Tris–HCl pH 8.0, 300 mM NaCl, 1% Triton X-100 and 5 mM DTT containing protease inhibitors (Roche). The insoluble material was solubilized in 50 mM Tris–HCl, pH 8.0, 8 M urea, 5 mM DTT and purified under denaturing conditions by anion exchange using Q Sepharose HP resin (Amersham) with elution over a NaCl gradient. The pH of the eluted sample was reduced by dialysis in 50 mM sodium acetate, pH 5.0, 8 M urea and 5 mM DTT and purification was completed by cation exchange using SP Sepharose HP resin (Amersham) with elution over a NaCl gradient. Urea used in the purification was freshly deionized by passage over AG 501-X8 (D) resin (Bio-Rad) in order to prevent protein carbamylation. The BRCA2 fragment was renatured by dialysis in 50 mM Tris–HCl, pH 8.0, 500 mM L-arginine and 10 mM DTT, and was subsequently transferred in to 20 mM Tris–HCl, 10% glycerol and 5 mM DTT by thorough dialysis. The sample was concentrated using an Amicon Ultra-4 centrifugal filter device and stored at −80°C. $BRCA2_{BRC1–8}$ protein was analysed by MALDI to exclude carbamylation by urea.

Full-length human RAD51 was cloned into a pET11d vector (Novagen), expressed in $recA^-$ cells BLR (DE3) competent cells (Novagen), and purified by a method described previously (20). Recombinant human RPA protein was made using the expressing vector p11d-tRPA (gift from Mark Wold) and purified in Dr Richard Wood's lab (Cancer Research UK Clare Hall Labs) as described previously (21).

Protein concentrations were estimated by UV absorbance at 280 nm (Varian Cary 50 spectrophotometer) with extinction coefficients estimated by ProtParam (22).

The BRC4 repeat peptide (KEPTLLGFHTASGKKVKTAKESLDKVKNLFDEKEQ) was synthesized at the Cancer Research UK Peptide Synthesis Unit, London. Stock solutions of the peptides dissolved in dH_2O and stored at −20°C were diluted appropriately for strand exchange assays.

Co-immunoprecipitation

Protein A–Sepharose 6 M (50 µl) (Sigma) was incubated with 20 µg rabbit polyclonal anti-RAD51 IgG (Santa Cruz Biotech) or with 20 µg non-immune rabbit serum IgG for 3 h at 4°C in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl and 1% Triton X-100. The resulting complexes were then incubated

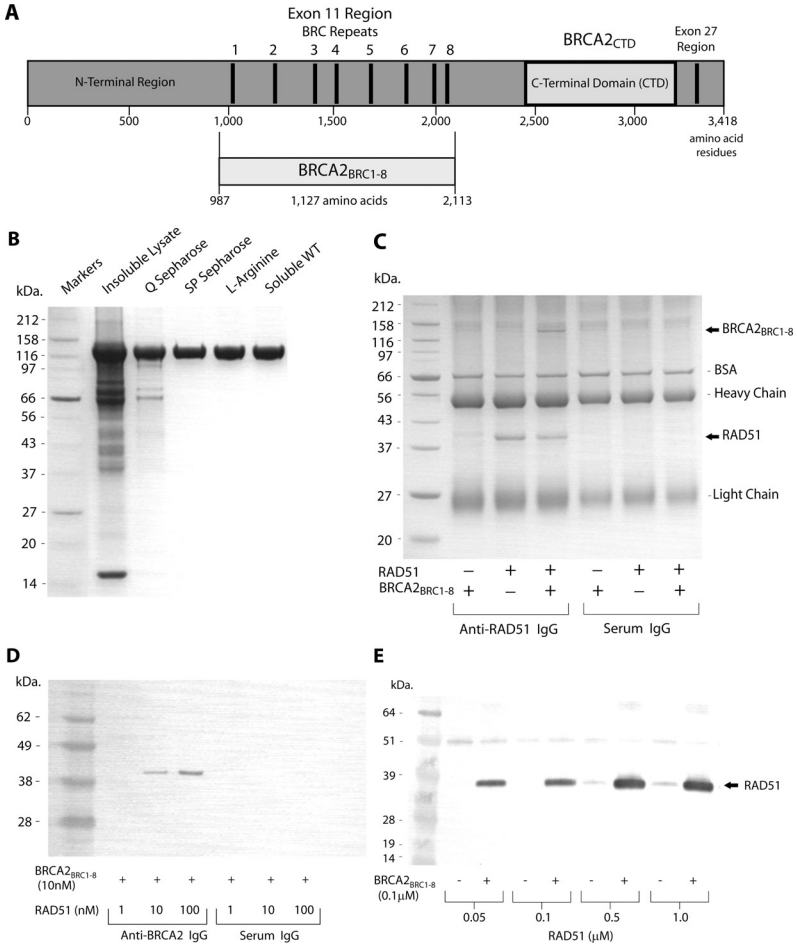

Figure 1. Expression and purification of a 1127 amino acid fragment of BRCA2 containing all 8 BRC repeats. (**A**) Human BRCA2 is a large protein of 3418 amino acids that can be divided into three general regions: an N-terminal region of unknown function, a central exon 11 region and a C-terminal DNA-binding domain (in addition to an extreme C-terminal RAD51-binding site encoded by exon 27). The eight RAD51-binding BRC repeats are located well spaced from one another amidst the long and divergent exon 11 region. We cloned and expressed a 1127 amino acid fragment of human BRCA2 corresponding to amino acid residues 987–2113 and spanning all 8 BRC repeats ($BRCA2_{BRC1–8}$). (**B**) The 126 kDa $BRCA2_{BRC1–8}$ protein was expressed in *E.coli* as an insoluble protein; it was purified under denaturing conditions by anionic exchange chromatography using Q Sepharose, and then cationic exchange chromatography using SP Sepharose. The resulting purified denatured sample was renatured in a buffer containing 500 mM L-arginine and was then soluble and stable after dialysis into a standard protein buffer. (**C**) Co-immunoprecipitation of purified $BRCA2_{BRC1–8}$ with human RAD51; purified BRCA2 and/or purified RAD51 were incubated with pre-formed complexes of protein A–Sepharose and rabbit polyclonal anti-RAD51 antibodies or non-immune rabbit serum IgG. The resulting complexes were visualized by SDS–PAGE and protein staining with SimplyBlue, showing that the purified $BRCA2_{BRC1–8}$ protein is functional in RAD51 binding. (**D**) An interaction between $BRCA2_{BRC1–8}$ and RAD51 is further confirmed by co-immunoprecipitation anti-BRCA2 antibodies (visualized by western blot using anti-RAD51 antibodies). $BRCA2_{BRC1–8}$ (10 nM) was incubated with 1–100 nM RAD51 before immunoprecipitation using pre-formed complexes of protein A–Sepharose and anti-BRCA2 or control irrelevant IgG. (**E**) The RAD51-binding capacity of $BRCA2_{BRC1–8}$ was tested through an immunoprecipitation of $BRCA2_{BRC1–8}$ (0.1 μM) with anti-BRCA2 antibodies, in the presence of 0.05, 0.1, 0.5 and 1.0 μM RAD51 (providing molar ratios of 0.5, 1, 5 and 10:1). Increasing amounts of RAD51 were co-immunoprecipitated at molar ratios between 0.5:1 and 5:1 (detected using anti-RAD51 antibodies), with binding saturation between 5:1 and 10:1. This suggests the presence of multiple RAD51-binding sites within each $BRCA2_{BRC1–8}$ molecule.

with the purified BRCA2 fragment (0.9 μM) and/or purified human RAD51 (0.9 μM) in the presence of 10 mg/ml BSA (Sigma) in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100 at 4°C for 3 h. The complexes were thoroughly washed in buffer containing 500 mM NaCl and subsequent pellets were suspended in loading buffer for analysis by SDS–PAGE (Invitrogen).

Co-immunoprecipitation reactions were also performed using anti-BRCA2 antibodies (Ab-1; Calbiochem) through the method described above but using 2 μg antibodies and with 10 nM $BRCA2_{BRC1–8}$ and 1 nm, 10 or 100 nM RAD51. Further reactions were performed using 2 μg anti-BRCA2 antibodies with 0.1 μM $BRCA2_{BRC1–8}$ and 0.05, 0.1, 0.5 or 1.0 μM RAD51. These reactions were visualized by western blot; samples were run on an SDS–PAGE gel (Invitrogen) and transferred on to nitrocellulose membrane (Invitrogen) at 25 mV for 90 min using transfer buffer 20 mM Tris, 150 mM glycine, 1% SDS and 20% methanol. The membrane was blocked in 50 ml blocking buffer [phosphate-buffered saline (PBS), 0.05% Tween and 10% milk powder] for 1 h at room temperature. It was then incubated with 2 μg mouse monoclonal anti-BRCA2 antibodies or 2 μg rabbit polyclonal anti-RAD51 IgG (Santa Cruz Biotech) in 20 ml blocking buffer for 1 h, before being washed three times in washing buffer (PBS and 0.05% Tween), each for 10 min. The membrane was then incubated with 2 μg alkaline phosphatase conjugated

anti-mouse antibody (Promega) or 2 μg alkaline phosphatase conjugated anti-rabbit antibody (Promega) in 20 ml blocking buffer for 1 h, before being washed three times in washing buffer (PBS and 0.05% Tween), each for 10 min. The blot was then developed by the addition of one SigmaFAST™ BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablet dissolved in 10 ml water.

Circular dichroism spectroscopy

The $BRCA2_{BRC1–8}$ sample was prepared for CD analysis by dialysis into 10 mM phosphate buffer (pH 7.5) and concentration was confirmed by absorbance at 280 nm, measured on a Varian Cary 50 spectrophotometer. CD spectra were obtained using a JASCO J-810 CD spectrometer. A native far UV CD spectrum was recorded over the range 185–260 nm using a 0.16 mg/ml sample in a cuvette of path length 0.05 cm. A denatured far UV CD spectrum was also recorded of a 0.16 mg/ml sample in the presence of 6 M guanidine–HCl (Fluka). CD data were converted from theta machine units to mean residue ellipticity and were deconvoluted using the CDSSTR algorithm of the DichroWeb server (23,24).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (13) either by pre-incubating RAD51 and $BRCA2_{BRC1–8}$ before incubation with radiolabelled DNA, or by adding $BRCA2_{BRC1–8}$ to pre-formed RAD51–DNA complexes. In the first instance, RAD51 (5 μM) was pre-incubated in the absence or presence of varying concentrations of $BRCA2_{BRC1–8}$ for 15 min at 37°C in Buffer A (see Strand Exchange Assay) containing KCl to a final concentration of 200 mM and 1 mM AMP-PNP. ApaL1 linearized ^{32}P-labelled dsϕX174 DNA (10 μM) was added, and the reaction was incubated at 37°C for a further 60 min. In the second instance, RAD51 (5 μM) was first incubated at 37°C for 5 min, before 10 μM of ApaL1 linearized ^{32}P-labelled dsϕX174 DNA was added, and the protein–DNA complex allowed to form for 10 min at 37°C before the addition of varying concentrations of $BRCA2_{BRC1–8}$. Reactions were incubated at 37°C for 60 min. Control reactions with DNA alone, or DNA plus $BRCA2_{BRC1–8}$ were incubated for 75 min at 37°C. The protein–DNA complexes were resolved by 0.5% agarose gel electrophoresis in TAE buffer. The dried gels were exposed to Phosphoimager screens and data processed on the Fuji FLA5000 scanner.

Ternary complex pull-down assay

5′-Biotinylated oligos (nt 5348–23) complementary to the ϕX174 (+) strand were annealed to ss ϕX174 (+) DNA at a ratio of 2:1, and the DNA-joint molecules were coupled to streptavidin-conjugated magnetic beads so as to contain the equivalent of 10 or 15 μM ss ϕX174 (+) DNA when incubated in a 10 μl reaction in Buffer A used for strand exchange reactions containing KCl to a final concentration of 200 mM. RAD51 (5 μM) in the absence or presence of the indicated amounts of $BRCA2_{BRC1–8}$ was pre-incubated in 10 μl of Buffer A at 37°C for 30 min before adding to streptavidin-conjugated magnetic beads bound to different amounts of the DNA-joint molecules. The mixture was incubated for a further 30 min at 37°C. Protein–DNA complexes bound to the beads were separated out of solution using a magnetic block. The supernatant was carefully removed into a fresh tube containing SDS–PAGE gel loading buffer. Protein–DNA complexes bound to the separated beads were resuspended in loading buffer. After heat denaturation in loading buffer, proteins were resolved by 4–12% SDS–PAGE, and revealed with SimplyBlue stain (Invitrogen).

Strand exchange assay

Strand exchange assays were carried out by a modification of a previously reported procedure (25). Reactions were assembled in reaction Buffer A (40 mM Tris–HCl, pH 7.8, 1 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 8 mM phosphocreatine and 25μg/ml creatine phosphokinase). The reactions were supplemented with either $(NH_4)_2SO_4$ (100 mM final concentration) or KCl (200 mM final concentration). RAD51, RPA, ssϕX174 DNA, $BRCA2_{BRC1–8}$ or the BRC4 peptide was included at the concentrations described in the text and figure legends. Reactions were incubated at 37°C as described before the addition of either non-radiolabelled or 5′-^{32}P-radiolabelled ApaL1 linearized-double-stranded (lds) ϕX174 DNA, and incubation at 37°C for a further 60 min. The reactions were stopped and de-proteinized by the addition of SDS/EDTA (0.2%/5 mM final concentration) and Proteinase K (600 μg/ml) and incubation at 37°C for a further 15 min. Reaction products were separated by 1% agarose gel electrophoresis in TAE buffer. The gels were either stained in ethidium bromide (0.5 μg/ml) and visualized under UV, or dried and exposed to Phosphoimager screens, with the results being analysed on a Fuji FLA-5000 scanner.

RESULTS

The expression and purification of $BRCA2_{BRC1–8}$

A 1127 amino acid fragment of human BRCA2 corresponding to amino acid residues 987–2113 and spanning all 8 BRC repeats ($BRCA2_{BRC1–8}$; Figure 1A) was expressed in *Escherichia coli* using a pET28a vector (Figure 1B). The 126 kDa protein was found to be highly over-expressed in the form of inclusion bodies. The $BRCA2_{BRC1–8}$ polypeptide was recovered from the insoluble fraction and purified under denaturing conditions through anionic and cationic exchange chromatography (Figure 1B). Once purified, the denatured sample was renatured in the presence of the refolding additive L-arginine; thereafter the $BRCA2_{BRC1–8}$ protein was found to be soluble and stable in standard protein buffers, thus allowing its use in biochemical investigations. The sequence of the purified $BRCA2_{BRC1–8}$ protein was confirmed and shown by mass spectrometry to be unaffected by possible chemical alteration due to the high concentrations of denaturant (data not shown). The protein was found to be functional in RAD51 binding through the co-immunoprecipitation of a RAD51–$BRCA2_{BRC1–8}$ complex using either anti-RAD51 (Figure 1C) or anti-BRCA2 (Figure 1D) antibodies. The RAD51-binding capacity of $BRCA2_{BRC1–8}$ was tested through RAD51–$BRCA2_{BRC1–8}$ co-immunoprecipitation using anti-BRCA2 antibodies over molar ratios from 0.5:1 up to 10:1 (Figure 1E). An increasing amount of RAD51 was co-immunoprecipitated between 0.5:1 and 5:1 molar

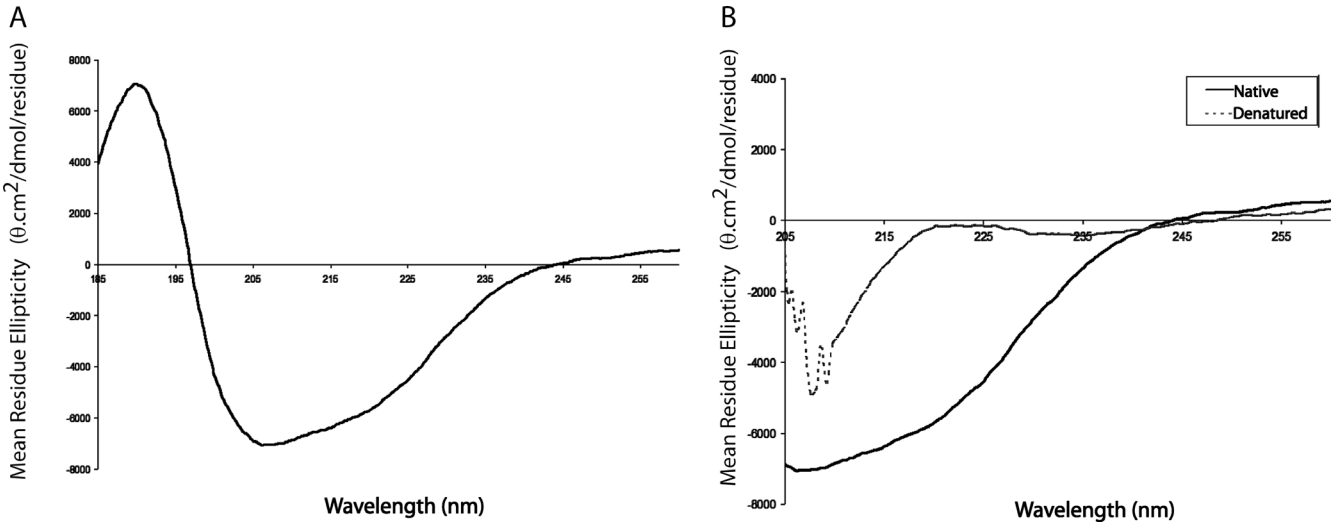

Figure 2. Circular dichroism spectroscopy of purified $BRCA2_{BRC1–8}$. (**A**) A far UV spectrum recorded over the range 185–260 nm is indicative of a mixture of secondary structural elements. Deconvolution of this spectrum using the CDSSTR algorithm indicates that the purified $BRCA2_{BRC1–8}$ protein consists of 9% α-helix, 33% β-sheet, 24% turns and 34% random coil (data were fitted with normalized r.m.s.d. of 0.031). This mixture of structured regions and flexible unstructured sequence is consistent with the predicted nature of the exon 11 region of BRCA2 given its generally poor conservation in terms of sequence but strong conservation in terms of inter-BRC repeat spacing between mammalian BRCA2 orthologues. (**B**) The differences between far UV spectra recorded over the range 205–360 nm under native and denaturing (6 M guanidine–HCl) conditions confirm that the renatured $BRCA2_{BRC1–8}$ sample does contain secondary structural elements that are destroyed upon denaturation.

ratios, with binding saturation observed between 5:1 and 10:1 molar ratios. This indicates the presence of multiple RAD51-binding sites, with each $BRCA2_{BRC1–8}$ molecule binding several RAD51 molecules.

Biophysical features of $BRCA2_{BRC1–8}$

In order to investigate the conformation of $BRCA2_{BRC1–8}$, the structure of the purified protein was probed by circular dichroism spectroscopy (Figure 2A). Far UV spectra comparing renatured and denatured samples of $BRCA2_{BRC1–8}$ revealed that the renatured sample does contain secondary structural elements including both α-helices and β-sheets (Figure 2B). Indeed, the secondary structure makeup of $BRCA2_{BRC1–8}$ was further defined through the deconvolution of a far UV spectrum over the range 185–240 nm. The sample was found to consist of 9% α-helix, 33% β-sheet, 24% turns and 34% random coil (data were fitted with normalized r.m.s.d. of 0.031). The finding that the $BRCA2_{BRC1–8}$ protein consists of 42% secondary structural elements and 58% turns or random coil suggests that whilst it contains some structured regions that are potentially of functional significance, it is also intrinsically flexible with much unordered sequence. It is important to note that the sequence of the exon 11 region displays in general only a moderate degree of conservation amongst closely related BRCA2 orthologues. Whilst the BRC repeats are themselves well conserved, the intervening sequences between consecutive repeats are remarkably poorly conserved. However, the length of the spacing they provide between BRC repeats is preserved. This is consistent with a portion of the BRCA2 exon 11 sequence constituting intrinsically unstructured flexible linkers rather than structured regions in their own right. The structural features of the renatured $BRCA2_{BRC1–8}$ protein are thus in keeping with the predicted nature of this region of human BRCA2 (5).

$BRCA2_{BRC1–8}$ forms a ternary complex with RAD51 and DNA

Isolated BRC repeats can either prevent RAD51 nucleoprotein filaments (11) or form a ternary complex with RAD51 on DNA (13). We tested the ability of $BRCA2_{BRC1–8}$–RAD51 complexes to bind DNA, or the effect of $BRCA2_{BRC1–8}$ on RAD51–DNA complexes, by EMSA. As expected, radiolabelled linear ϕX174 dsDNA (Figure 3, lane 1) was retarded in its mobility by addition of RAD51, marking formation of a nucleoprotein filament (lane 2). Interestingly, pre-formed $BRCA2_{BRC1–8}$–RAD51 complexes induce a further, concentration-dependent retardation in the mobility of the radiolabelled dsDNA (lanes 3–9). An apparent plateau in retardation occurs at >2.8 μM of $BRCA2_{BRC1–8}$ that may reflect saturation of binding. Addition of increasing amounts of $BRCA2_{BRC1–8}$ to pre-formed RAD51–DNA complexes (lanes 10–16) reveals a similar retardation in the mobility of the radiolabelled dsDNA. Retardation peaks at 1.7–3.3 μM $BRCA2_{BRC1–8}$, with a slight decrease in retardation at 3.9 μM, whose significance could not readily be ascertained, because the $BRCA2_{BRC1–8}$ fragment could not be purified at higher concentrations. We speculate, however, that this biochemical feature could reflect the proposal that BRCA2 may not only deliver RAD51 for filament formation at sites of recombinational repair, but also help to dissolve RAD51 filaments following the completion of repair (16). $BRCA2_{BRC1–8}$ alone does not retard the mobility of radiolabelled dsDNA (lanes 17 and 18), indicating that complex formation with DNA occurs via RAD51. Thus, irrespective of the order of addition, our findings are indicative of a ternary complex in which $BRCA2_{BRC1–8}$ binds to the RAD51–DNA nucleoprotein filament.

Further evidence for ternary complex formation comes from affinity chromatography (Figure 4). A short, 73 bp

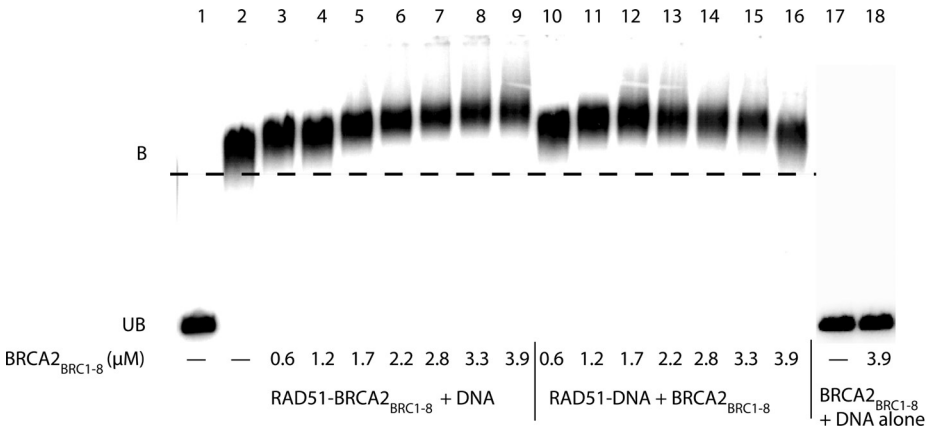

Figure 3. EMSA analysis of ternary complex formation between $BRCA2_{BRC1–8}$, RAD51 and DNA. The $BRCA2_{BRC1–8}$ fragment induces a concentration-dependent shift in the mobility of RAD51/DNA complexes (lane 2) regardless of whether radiolabelled DNA is added to a pre-formed RAD51–$BRCA2_{BRC1–8}$ complex (lanes 3–9), or $BRCA2_{BRC1–8}$ is added to a pre-formed RAD51 filament on radiolabelled DNA (lanes 10–16). The dashed line marks the lower edge of RAD51–DNA complexes (marked B for bound). The first lane shows the migration of radiolabelled dsDNA without added protein (marked UB for unbound). Note that $BRCA2_{BRC1–8}$ alone has no effect on the mobility of the radiolabelled dsDNA (lanes 17 and 18).

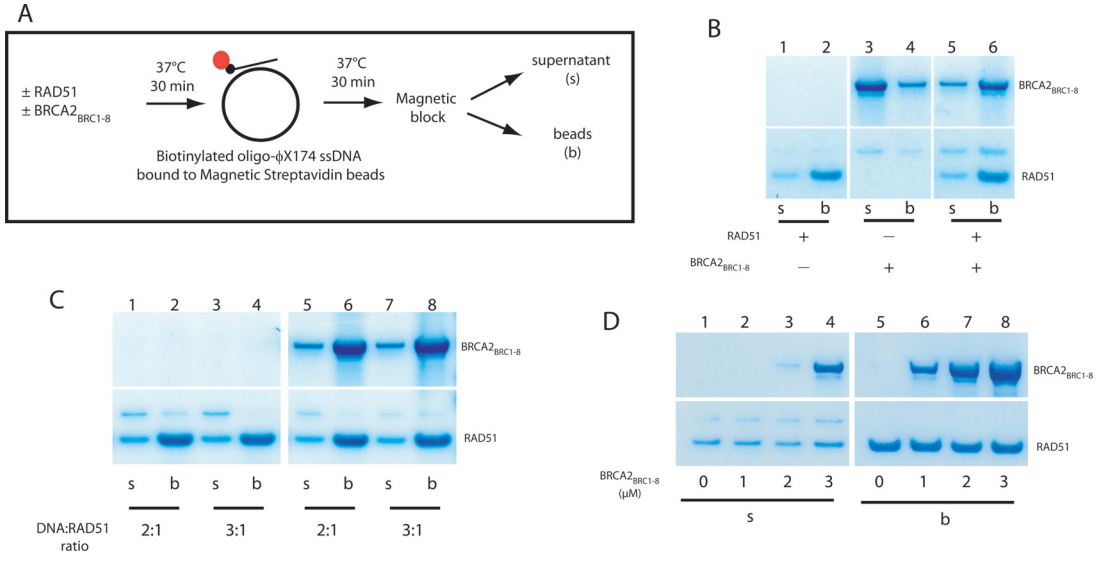

Figure 4. $BRCA2_{BRC1–8}$ forms a ternary complex with RAD51 on DNA-joint molecules. (**A**) Schematic representation of the binding assay. Proteins were pre-incubated in strand exchange buffer containing 1 mM AMP-PNP (Materials and Methods) at 37°C for 30 min, before the addition to different concentrations of DNA-joint molecules bound to streptavidin-conjugated magnetic beads, and incubation for a further 30 min at 37°C. Proteins in the supernatant (s) were separated from the protein–DNA complexes bound to the magnetic beads (b) before analysis by SDS–PAGE. (**B**) Binding of $BRCA2_{BRC1–8}$ is dependent on RAD51. SDS–PAGE analysis of proteins in the supernatant (s) or bound to DNA-joint molecules (b) is shown. DNA-joint molecules were incubated with RAD51 (5 μM) alone (lanes 1 and 2), $BRCA2_{BRC1–8}$ (2 μM) alone (lanes 3 and 4) or RAD51 with $BRCA2_{BRC1–8}$ (lanes 5 and 6). (**C**) Ternary complex formation between $BRCA2_{BRC1–8}$ and RAD51 bound to DNA-joint molecules occurs at different ssDNA/RAD51 concentration ratios. Complex formation by RAD51 (5 μM) is shown at ssDNA/RAD51 ratios of 2:1 or 3:1 in the absence (lanes 1–4) or presence (lanes 5–8) of $BRCA2_{BRC1–8}$ (2 μM). (**D**) Ternary complex formation by RAD51 (5 μM) and DNA-joint molecules at an ssDNA/RAD51 ratio of 3:1 with increasing concentrations of $BRCA2_{BRC1–8}$. Lanes 1 and 5 represent control reactions performed in the absence of $BRCA2_{BRC1–8}$. The (s) and (b) fractions are indicated.

biotinylated-ssDNA oligonucleotide was annealed to a homologous circular 5386 bp ϕX174 ssDNA molecule in order to create a biotinylated substrate, which predominantly represents a ssDNA circle (Figure 4A). RAD51 and $BRCA2_{BRC1–8}$ (either alone or together, as indicated) were then incubated with the DNA substrate. Proteins bound to the DNA-joint molecule (labelled 'b' in the figure) were isolated by affinity chromatography on magnetic-streptavidin beads, resolved by SDS–PAGE, and revealed by protein staining. Unbound proteins left behind in the supernatant (labelled 's' in the figure) were compared with proteins bound to the DNA-joint molecules.

As expected, RAD51 alone binds efficiently to DNA-joint molecules (Figure 4B, lanes 1 and 2), marking the formation of a nucleoprotein filament. In the absence of RAD51, the majority of $BRCA2_{BRC1–8}$ remains in the supernatant

(Figure 4B, lane 3), whilst a small amount of $BRCA2_{BRC1–8}$ binds to the DNA substrate (Figure 4B, lane 4). Since similar levels of $BRCA2_{BRC1–8}$ bind to the streptavidin beads alone (data not shown), $BRCA2_{BRC1–8}$ pull-down in this case likely reflects a non-specific interaction with the beads rather than DNA binding. Importantly, when RAD51 is present, the majority of $BRCA2_{BRC1–8}$ is brought down with the DNA-joint molecule (Figure 4B, compare lanes 3 and 4 with 5 and 6). Ternary complex formation between $BRCA2_{BRC1–8}$, RAD51 and DNA was observed over a range of nucleotide/protein concentration ratios, including 1:1 (data not shown), 2:1 and 3:1 (Figure 4C), at which RAD51 has been shown previously to perform strand exchange *in vitro* (26).

This conclusion is further substantiated by the concentration-dependent binding of $BRCA2_{BRC1–8}$ to a fixed amount (5 μM) of the RAD51–DNA-joint molecule complex formed at a nucleotide/protein concentration ratio of 1:1 (Figure 4D). At 1 μM concentration, $BRCA2_{BRC1–8}$ is entirely bound to the RAD51–DNA complex (compare lanes 2 and 6), further confirming the results in Figure 4B. Increasing amounts of $BRCA2_{BRC1–8}$ are pulled down with the RAD51–DNA complex, reaching saturation at ~3 μM (compare lanes 3 and 7 versus 4 and 8). This is similar to the results of EMSA shown in Figure 3B, where saturation of binding occurs at 2.8–3.9 μM. Each $BRCA2_{BRC1–8}$ molecule contains multiple BRC repeats potentially capable of binding RAD51 with varying affinities (8,12). Thus, the saturation we observe as the concentration of $BRCA2_{BRC1–8}$ is increased could reflect intermolecular competition for binding to limiting amounts of RAD51, with only those BRC repeats in each $BRCA2_{BRC1–8}$ molecule that have the highest affinity for RAD51 able to bind when the $BRCA2_{BRC1–8}$–RAD51 molar ratio approaches 1:1.

Taken together, the findings we have presented in Figures 3 and 4 provide direct evidence that the multiple BRC repeat region of BRCA2 can form a ternary complex with RAD51 on DNA rather than disrupting it. This is in contrast to the apparently inhibitory effect of individual BRC repeats on RAD51 nucleoprotein filament formation [(11) and data not shown), suggesting that function of BRC repeats is modified and directed when present as an eight-repeat unit within the context of the exon 11 region.

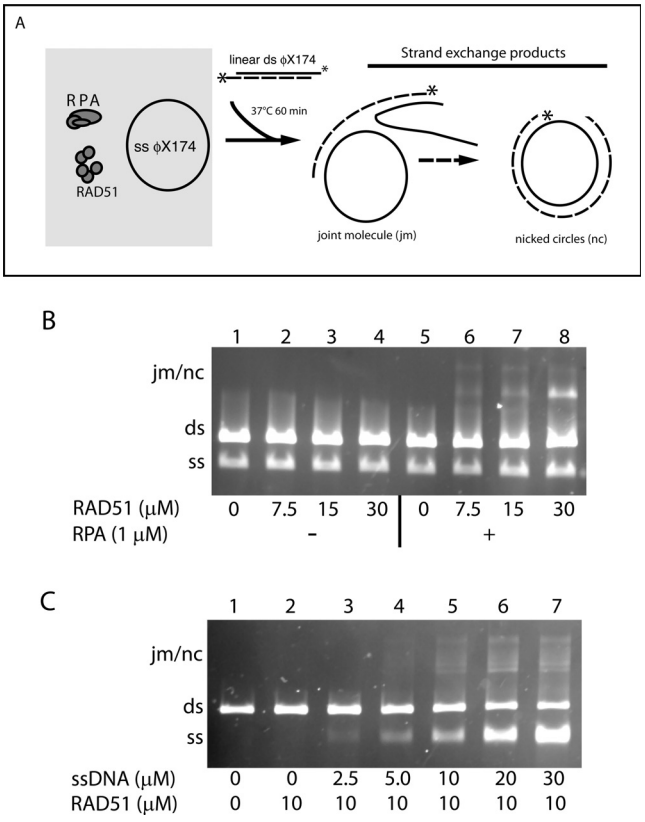

Figure 5. RAD51-mediated strand exchange assay. (**A**) Schematic representation of the strand exchange assay. (**B**) Stimulation of RAD51-mediated strand exchange in the presence of $(NH_4)_2SO_4$ requires RPA. Reaction mixtures containing 100 mM $(NH_4)_2SO_4$ were pre-incubated without (lanes 1 and 5) or with increasing concentration of RAD51 (lanes 2–4 and 6–8) and ssDNA (30 μM) for 5 min followed by buffer control (lanes 1–4, no RPA) or with RPA (1 μM; lanes 5–8) for another 5 min. Linear dsDNA (30 μM) was added to stimulate strand exchange for a further 60 min. Reactions were performed at 37°C. The reactions were de-proteinized and analysed by gel electrophoresis and ethidium bromide staining. (**C**) Stimulation of RAD51-mediated strand exchange as function of ssDNA titration. Reactions were set up in the presence of $(NH_4)_2SO_4$ and RPA as described in (B) except that the RAD51 concentration was constant at 10 μM (lanes 2–7) in the absence (lanes 1 and 2) or presence (lanes 3–7) of increasing concentration of ssDNA. Linear dsDNA was present at 10 μM. The reactions products were analysed as described previously. The ssDNA/RAD51 ratio is as indicated.

$BRCA2_{BRC1–8}$ promotes RAD51-mediated strand exchange

The recombination function of RAD51 may be demonstrated through its ability to perform strand exchange between circular ssDNA and linearized dsDNA (radiolabelled and non-radiolabelled) in the presence of ssDNA-binding protein, RPA (25–27) (Figure 5A). The strand exchange assay is now well established as a method of demonstrating recombination *in vitro*, and has been used to study the recombination functions of all RAD51 recombinases. Interestingly, whilst *E.coli* RecA and yeast RAD51 are both highly active in recombination, mammalian RAD51 is a relatively poor recombinase when isolated *in vitro* (28–32). Clearly, co-factors present in mammalian cell nuclei are required to promote RAD51-mediated recombination that are not necessary for the recombinase function of yeast and bacterial RAD51 homologues.

In order to study RAD51-mediated strand exchange outside its normal cellular environment, buffer systems have been developed that optimally stimulate RAD51 recombinase activity *in vitro* (25,33,34). Importantly, the addition of different ionic species stimulates the recombinase activity of mammalian RAD51. It has been reported that the inclusion of either $(NH_4)_2SO_4$ or Ca^{2+} in the reaction buffer enhances RAD51-mediated strand exchange *in vitro* (25,33,34). This has provided a useful tool for investigating the recombinase activity of mammalian RAD51, which would otherwise be difficult to study. Consistent with these previous reports, we find that in the presence of ammonium sulphate, RAD51 mediates strand exchange in a concentration-dependent manner with respect to DNA (Figure 5B and C) that is absolutely dependent on RPA as has been reported previously (25).

Similarly, RAD51 is effective at strand exchange in the presence of 0.3–0.6 mM Ca^{2+}, also as reported previously (data not shown).

However, the conditions used in these *in vitro* assays for RAD51-mediated strand exchange do not conform to the ionic milieu found within the mammalian cell nucleus, in which K^+ and Mg^{2+} are the predominant ionic species (35–38). Indeed, there is structural evidence that K^+ may modulate the ATPase activity of an archael homologue of RAD51 (39). Accordingly, we performed experiments under approximately physiological conditions (200 mM K^+ and 1 mM Mg^{2+}), in the absence of ammonium or calcium ions, and in the presence of an ATP regenerating system (34).The concentration of RPA in the reaction was also adjusted so that the need for the $BRCA2_{CTD}$, which is required to displace RPA, was obviated (18,40).

At ssDNA/RAD51 ratio of 3:1, RAD51 was found to weakly stimulate strand exchange, as has been reported previously (Figure 6A, lane 1) (34). However, upon the addition of $BRCA2_{BRC1–8}$, RAD51-mediated strand exchange was robustly stimulated in a dose-dependent manner, with the formation of joint molecule and complete recombination products in the form of nicked circles (Figure 6A, lanes 2–4). $BRCA2_{BRC1–8}$ stimulated strand exchange when present at one-fifth of the concentration of RAD51 (Figure 6A, lane 2). Efficient formation of a ternary complex with RAD51 and DNA is observed at a non-saturating concentration of $BRCA2_{BRC1–8}$ (Figure 4D, lane 6) which would suggest that the binding of RAD51 is thus likely to involve multiple BRC repeats of each $BRCA2_{BRC1–8}$ molecule. Stimulation of strand exchange by $BRCA2_{BRC1–8}$ is also clearly dependent upon the presence of Mg^{2+} and ATP (Figure 6B).

We observe a stimulatory effect of ~3 μM $BRCA2_{BRC1–8}$ on RAD51-mediated strand exchange, equating to ~24 μM of single BRC repeats. However, under the same conditions, an equivalent concentration of isolated BRC4 peptide inhibits, rather than promotes, strand exchange (Figure 6C), consistent with its ability to inhibit RAD51 nucleoprotein formation [(11) and data not shown]. This suggests that the eight BRC repeats, when organized in the context of the $BRCA2_{BRC1–8}$ fragment, may possess an activity not shared with isolated BRC peptides. However, given the differences in sequence between the different BRC repeats in human BRCA2, which are poorly characterized in terms of function, further investigation is warranted.

Another ion found in the mammalian nucleus is Ca^{2+}, which is reportedly present at concentrations of ~0.1 μM free Ca^{2+} (41), although higher local concentrations might be achieved during biological signalling. Although Ca^{2+} alone at 0.3–0.6 mM can stimulate RAD51-mediated strand exchange *in vitro* [(33) and data not shown], we find that when physiological concentrations of K^+ and Mg^{2+} are also present, RAD51 activity is relatively low (Figure 6D, compare lane 1 with lanes 2–5). However, under these conditions, a sub-stoichiometric amount of $BRCA2_{BRC1–8}$ provided a substantial stimulus to the strand exchange reaction (Figure 6D, compare lane 6 with lanes 7–10). Thus, in an ionic milieu approaching the physiological conditions of the mammalian nucleus, we find that whilst recombinase activity of RAD51 diminishes, the level of stimulation achieved by $BRCA2_{BRC1–8}$ greatly increases.

We have demonstrated that, at the 3:1 DNA/RAD51 ratio used in the strand exchange assays, $BRCA2_{BRC1–8}$ forms a ternary complex involving RAD51 and ssDNA (Figures 3 and 4), which plausibly represents the species active in stimulating strand exchange. However, to rule out the possibility that $BRCA2_{BRC1–8}$ promotes strand exchange by sequestering RAD51 away from the linear dsDNA used in these reactions, we examined the ability of RAD51 to promote strand exchange—in the absence or presence of an increasing concentration of $BRCA2_{BRC1–8}$—at different DNA/RAD51 ratios ranging from 1:1 to 5:1 (Figure 6E). If $BRCA2_{BRC1–8}$ were simply serving as a sink for free RAD51, this experiment should reveal an increase in strand exchange by RAD51 alone as the DNA/RAD51 ratio increases. This does not occur. Instead, we find that $BRCA2_{BRC1–8}$ stimulates strand exchange best when the DNA/RAD51 ratio is between 1:1 and 3:1, the same optimum reported with RAD51 alone, but under different ionic conditions in which species such as NH_4^+ (25) and Ca^{2+} (33) substitute for $BRCA2_{BRC1–8}$.

Collectively, our findings have two important implications. First, they highlight an interesting difference between the high-RAD51 activity under the relatively non-physiological conditions so far used *in vitro* and its markedly diminished capacity to mediate strand exchange in ionic conditions more typical of the mammalian nucleus. We infer from our work that *in vivo*, additional co-factors such as BRCA2 are essential for efficient homologous recombination mediated by RAD51. This is entirely consistent with the cellular effects of BRCA2 inactivation on DNA repair or chromosomal integrity (1–3), and on homology-mediated gene conversion (4,42).

Moreover, the experiments we report here demonstrate a role for the multi-BRC repeat region conserved in all mammalian BRCA2 molecules, which is independent of the $BRCA2_{CTD}$. The DNA-binding $BRCA2_{CTD}$ has been shown to stimulate RAD51-mediated strand exchange *in vitro*; its role is believed to involve a displacement of RPA from single-stranded DNA in order to allow RAD51 to bind (18,40,43). However, we find that when RPA concentrations are lowered to obviate the need for its displacement by the $BRCA2_{CTD}$, a previously unrecognized activity of $BRCA2_{BRC1–8}$ in directly promoting RAD51-mediated strand exchange is revealed. Thus, our findings also establish a biological function for the evolutionarily conserved region of BRCA2 containing the eight BRC repeats.

DISCUSSION

In this paper we describe a significant advance in understanding the role of mammalian BRCA2 in homologous recombination. We have expressed and purified a 1127 amino acid region of human BRCA2 containing all 8 BRC repeats ($BRCA2_{BRC1–8}$), and have demonstrated that as a collective unit, the multiple BRC repeats can associate in a ternary complex with RAD51 on ssDNA and dsDNA. Ternary complex formation and the stimulation of RAD51-mediated strand exchange occur at sub-stoichiometric concentrations of $BRCA2_{BRC1–8}$ relative to RAD51, indicating that each $BRCA2_{BRC1–8}$ molecule probably binds several molecules of RAD51.

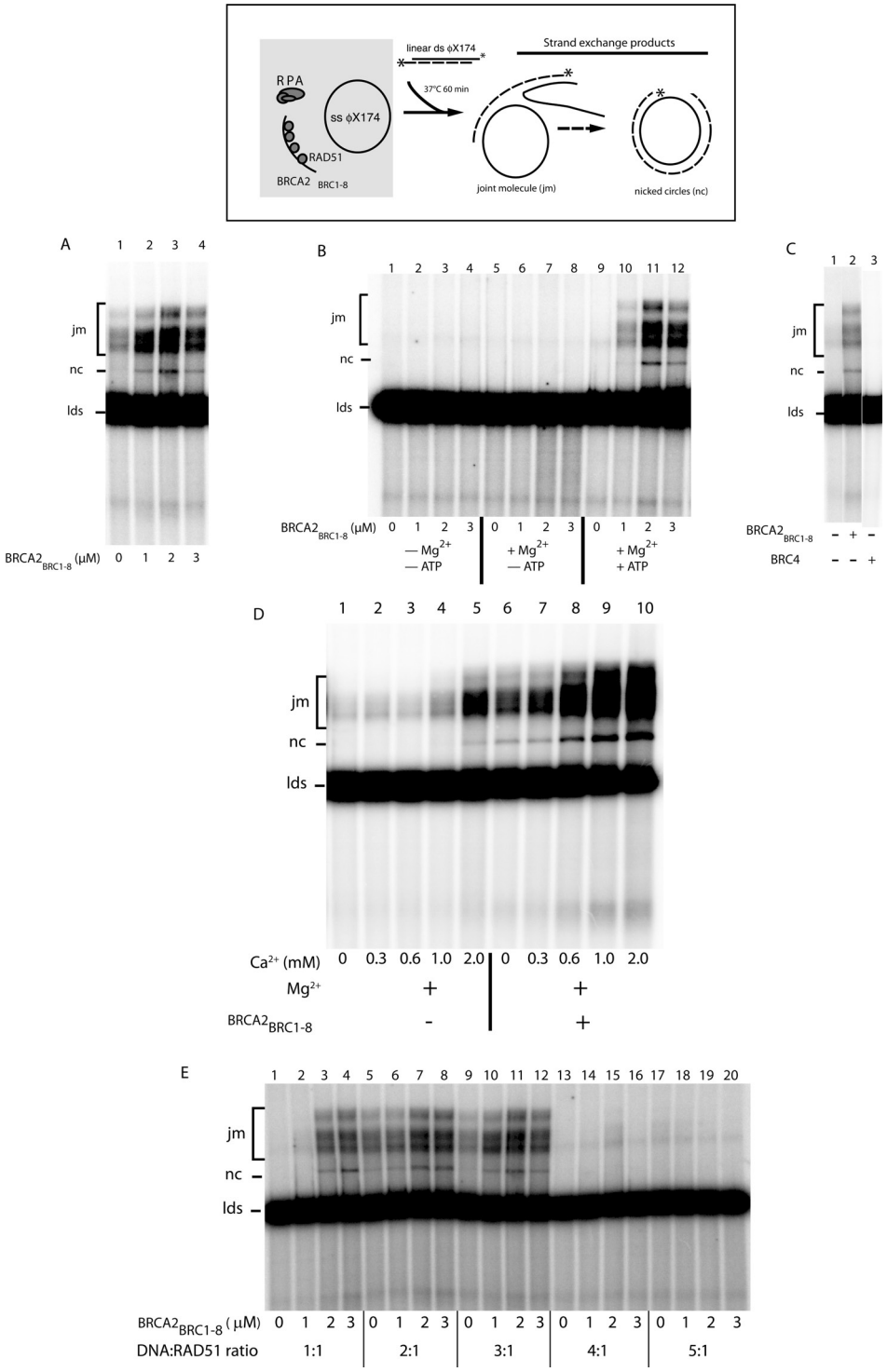

Figure 6. Stimulation of RAD51-mediated strand exchange by $BRCA2_{BRC1–8}$. (**A**) Stimulation of RAD51-mediated strand exchange by $BRCA2_{BRC1–8}$ in the presence of KCl and Mg^{2+}. Reactions were carried out using ssDNA and RAD51 at a ratio of 3:1. RAD51 was pre-incubated without (lane 1) or with increasing concentrations of $BRCA2_{BRC1–8}$ (lanes 2–4) for 5 min before adding ssDNA and RPA (0.26 μM). After a further 5 min incubation, radiolabelled linear dsDNA (10 μM) was added and incubated for a further 60 min. The reactions steps were incubated at 37°C. The strand exchange products were analysed by gel electophoresis and phosphoimager. (**B**) Stimulation of RAD51-mediated strand exchange by $BRCA2_{BRC1–8}$ is dependent on Mg^{2+} and ATP. Reactions were carried out as described in (A) either in the absence of both co-factors Mg^{2+} and ATP (lanes 1–4), in the absence of ATP but presence of Mg^{2+} (lanes 5–8) or in the presence of both co-factors (lanes 9–12). $BRCA2_{BRC1–8}$ was present as indicated. (**C**) Effect of $BRCA2_{BRC1–8}$ (lane 2) or isolated BRC4 peptide (lane 3) on RAD51-mediated strand exchange when BRC repeats are present at equivalent molarities. Reactions were carried out as in (A). The first lane shows a reaction without added proteins. (**D**) The effect of $BRCA2_{BRC1–8}$ on RAD51-mediated strand exchange is not dependent on physiologically relevant Ca^{2+} concentrations. Reactions are as described in (B) except that RAD51, either in the absence (lanes 1–5) or presence of $BRCA2_{BRC1–8}$ (2 μM; lanes 6–10), was incubated with buffer containing no Ca^{2+} ions (lanes 1 and 6) or an increasing concentration of Ca^{2+} (lanes 2–5 and 7–10). (**E**) $BRCA2_{BRC1–8}$ stimulates RAD51-mediated strand exchange at different DNA/RAD51 ratios. Reactions are as described in (A).

We have further shown that $BRCA2_{BRC1–8}$ stimulates RAD51-dependent strand exchange under physiologically relevant conditions in which human RAD51 shows only poor recombinase activity. Crucially, our description of a novel function for the BRC repeat region of mammalian BRCA2, does not depend on the $BRCA2_{CTD}$. Previous work had shown stimulation of strand exchange by the *Mus musculus* $BRCA2_{CTD}$, the *U.maydis* BRCA2 homologue Brh2 containing a CTD and single BRC repeat and the human BRC3–4 fused to a CTD (18,40,43). Our results establish $BRCA2_{BRC1–8}$ as a co-factor necessary to promote RAD51-dependent homologous recombination under physiological conditions resembling those present in the nuclei of living cells, consistent with the known importance of BRCA2 to homologous recombination *in vivo*.

Our data are consistent with a model (11,16) inferred from the observations that BRCA2 is complexed to RAD51 even before DNA damage (44) and is required to target RAD51 to sites of DNA damage (2,14,16) protected by RPA (45) in preparation for homologous recombination (Figure 7). Thus, BRCA2 is expected to function both in sequestering RAD51 to prevent promiscuous filament formation in undamaged cells, and in targeting and promoting RAD51's recombinase function at sites of DNA damage, perhaps as a result of activation by post-translational modifications (15,16). At sites of damage, BRCA2 is likely to perform multiples functions in promoting RAD51-mediated homologous recombination (Figure 7). The displacement of RPA by the $BRCA2_{CTD}$ may arrange the exon 11-BRC repeat-bound RAD51 molecules in a manner conducive to their loading on to damaged DNA for nucleoprotein filament formation (Figure 7). Indeed, whilst the *in vitro* strand exchange reaction presented here does not depend on the DNA-binding $BRCA2_{CTD}$, it is likely that *in vivo,* the CTD helps, not only to target the active BRCA2–RAD51 complex to junctions between ssDNA and dsDNA (18) but also to orientate the complex with respect to the polarity of the resected double-strand break.

Our proposal for the function of the multiple BRC repeats within the exon 11 region is supported by the finding that under specific conditions, BRC3 and BRC4 can associate with a nucleoprotein filament without causing its disruption (13). Given the known antagonism between the BRC repeat–RAD51 interaction and RAD51 oligomerization, this might conceivably be explained by BRC repeats possessing two modes of RAD51 binding, one that is inhibitory to filament formation (12) and another that is compatible with filament formation (13), reflecting the duality of the function of BRCA2 in regulating RAD51 activity during homologous recombination (11,15). BRC repeats may thus interact with a RAD51 filament as a ternary complex if present in sub-stoichiometric amounts, but then disrupt the filament through their inhibitory binding mode if present in excess (11,13,40). The large exon 11 sequence that houses the eight BRC repeats would thus be expected to control the binding mode of the BRC repeats, allowing them to form a ternary complex with RAD51 and DNA, as described in this study.

However, it is also possible that the inhibition and the stimulation of RAD51 activity by BRCA2 reflect only a single mode of BRC repeat-RAD51 binding, and not two distinct modes. One major difficulty with the hypothesis of a second mode of binding of the BRC repeat to RAD51 (13) lies in the observation that the amino acid conservation within BRC repeats is already accounted for by the known inhibitory interaction between BRC repeats and RAD51 through mimicry of self-association (12,19,46). It would therefore be surprising for a second RAD51-binding mode

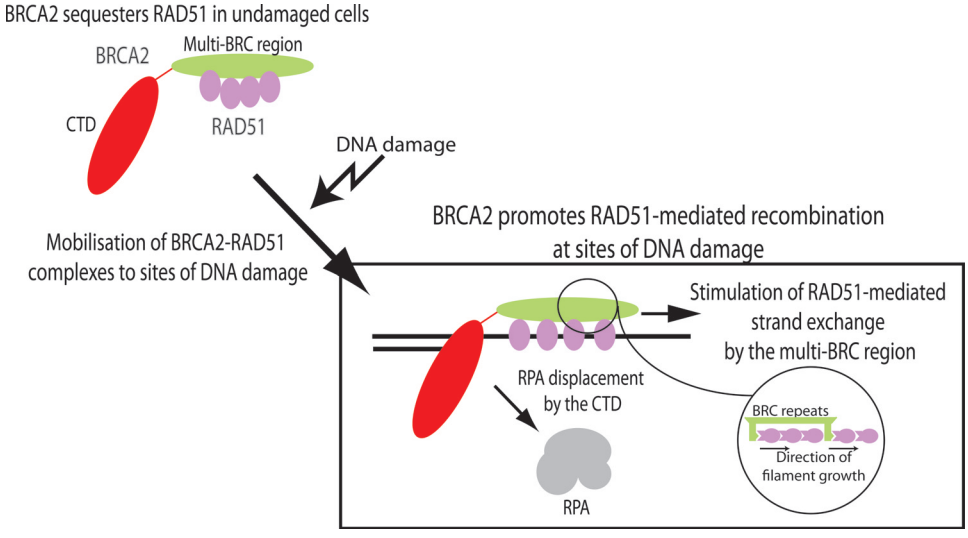

Figure 7. A model for the multiple roles of BRCA2 in regulating RAD51-mediated homologous recombination BRCA2 sequesters RAD51 in an inactive complex in undamaged cells. Following DNA damage, the activated BRCA2–RAD51 complex is targeted via the C-terminal domain (CTD) to the linear-duplex DNA of the RPA-coated, resected single-stranded ends. The CTD is essential to displace RPA, enabling the BRCA2–RAD51 complex to bind DNA to form the ternary complex demonstrated here (Figures 3 and 4). This may help to orient the BRCA2–RAD51 complex with respect to the polarity of the resected double-strand break. Our results (Figure 6) suggest that the multi-BRC repeat region of BRCA2 can stimulate RAD51-mediated strand exchange, distinct from the RPA-displacing activity of the CTD, a novel activity that promotes homologous recombination. We speculate that stimulation of strand exchange involves the same mode of RAD51–BRC repeat binding as in the inactive complex, with each BRC repeat capping one self-association surface of RAD51, but permitting filament growth from its opposite surface (circled inset). The resulting nucleoprotein filament might thus consist of multiple RAD51 multimers separated by interleaved BRC repeats.

to have emerged for BRC repeats with a similar pattern of sequence conservation as that of the first binding mode.

We therefore favour an alternative explanation for the stimulation of RAD51-mediated strand exchange by BRCA2, which does not invoke a second binding mode. We envision that the BRC repeats of $BRCA2_{BRC1–8}$ bind RAD51 in only one mode, inhibitory of RAD51 oligomerization, with each BRC repeat capping one self-association surface of RAD51, but permitting filament growth from its opposite surface (Figure 7 circled inset). The resulting nucleoprotein filament would thus consist of multiple filaments separated by interleaved BRC repeats. Indeed, the long exon 11 sequence between adjacent BRC repeats would allow for considerable growth of each capped filament. It is important to note, however, that our results are consistent with either of these structural possibilities explaining the novel activity of $BRCA2_{BRC1–8}$ in promoting RAD51-mediated strand exchange.

In summary, we have established a unique tool for research into mammalian homologous recombination through the expression and purification of $BRCA2_{BRC1–8}$, a fragment of BRCA2 containing all eight BRC repeats. We report that within the context of the wider sequence provided by $BRCA2_{BRC1–8}$, the eight BRC repeats exert a previously unrecognized, collective function in promoting RAD51-mediated strand exchange through ternary complex formation, independent of the BRCA2 C-terminal domain. Thus, our work provides direct biochemical evidence that human BRCA2 can function as a recombination mediator—a critical but unresolved issue (17). The identification of a mechanism for the stimulation of RAD51-mediated recombination by the multiple BRC repeat-containing region of human BRCA2 provides an important insight into the process of recombinational DNA repair in mammalian cells.

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Conflict of interest statement. None declared.

REFERENCES

1. Patel,K.J., Yu,V.P., Lee,H., Corcoran,A., Thistlethwaite,F.C., Evans,M.J., Colledge,W.H., Friedman,L.S., Ponder,B.A. and Venkitaraman,A.R. (1998) Involvement of Brca2 in DNA repair. *Mol. Cell*, **1**, 347–357.
2. Yu,V.P., Koehler,M., Steinlein,C., Schmid,M., Hanakahi,L.A., van Gool,A.J., West,S.C. and Venkitaraman,A.R. (2000) Gross chromosomal rearrangements and genetic exchange between nonhomologous chromosomes following BRCA2 inactivation. *Genes Dev.*, **11**, 1400–1406.
3. Connor,F., Bertwistle,D., Mee,P.J., Ross,G.M., Swift,S., Grigorieva,E., Tybulewicz,V.L. and Ashworth,A. (1997) Tumorigenesis and a DNA repair defect in mice with a truncating BRCA2 mutation. *Nature Genet.*, **17**, 423–430.
4. Moynahan,M.E., Pierce,A.J. and Jasin,M. (2001) BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol. Cell*, **2**, 511–518.
5. Pellegrini,L. and Venkitaraman,A. (2004) Emerging functions of BRCA2 in DNA recombination. *Trends Biochem. Sci.*, **29**, 310–316.
6. West,S.C. (2003) Molecular views of recombination proteins and their control. *Nature Rev. Mol. Cell. Biol.*, **4**, 435–445.
7. Bork,P., Blomberg,N. and Nilges,M. (1996) Internal repeats in the BRCA2 protein sequence. *Nature Genet.*, **13**, 22–26.
8. Wong,A.K., Pero,R., Ormonde,P.A., Tavtigian,S.V. and Bartel,P.L. (1997) RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. *J. Biol. Chem.*, **272**, 31941–31944.
9. Bignell,G., Micklem,G., Stratton,M.R., Ashworth,A. and Wooster,R. (1997) The BRC repeats are conserved in mammalian BRCA2 proteins. *Hum. Mol. Genet.*, **6**, 53–58.
10. Kojic,M., Kostrub,C.F., Buchman,A.R. and Holloman,W.K. (2002) BRCA2 homolog required for proficiency in DNA repair, recombination, and genome stability in *Ustilago maydis*. *Mol. Cell*, **10**, 683–691.
11. Davies,A.A., Masson,J.Y., McIlwraith,M.J., Stasiak,A.Z., Stasiak,A., Venkitaraman,A.R. and West,S.C. (2001) Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. *Mol. Cell*, **7**, 273–282.
12. Pellegrini,L., Yu,D.S., Lo,T., Anand,S., Lee,M., Blundell,T.L. and Venkitaraman,A.R. (2002) Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. *Nature*, **420**, 287–293.
13. Galkin,V.E., Esashi,F., Yu,X., Yang,S., West,S.C. and Egelman,E.H. (2005) BRCA2 BRC motifs bind RAD51-DNA filaments. *Proc. Natl Acad. Sci. USA*, **102**, 8537–8542.
14. Chen,C.F., Chen,P.L., Zhong,Q., Sharp,Z.D. and Lee,W.H. (1999) Expression of BRC repeats in breast cancer cells disrupts the BRCA2–Rad51 complex and leads to radiation hypersensitivity and loss of G_2/M checkpoint control. *J. Biol. Chem.*, **274**, 32931–32935.
15. Venkitaraman,A.R. (2002) Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell*, **108**, 171–182.
16. Yu,D.S., Sonoda,E., Takeda,S., Huang,C.L., Pellegrini,L., Blundell,T.L. and Venkitaraman,A.R. (2003) Dynamic control of Rad51 recombinase by self-association and interaction with BRCA2. *Mol. Cell*, **12**, 1029–1041.
17. Sung,P. (2005) Mediating repair. *Nature Struct. Mol. Biol.*, **12**, 213–214.
18. Yang,H., Jeffrey,P.D., Miller,J., Kinnucan,E., Sun,Y., Thoma,N.H., Zheng,N., Chen,P.L., Lee,W.H. and Pavletich,N.P. (2002) BRCA2 function in DNA binding and recombination from a BRCA2–DSS1–ssDNA structure. *Science*, **297**, 1837–1848.
19. Lo,T., Pellegrini,L., Venkitaraman,A.R. and Blundell,T.L. (2003) Sequence fingerprints in BRCA2 and RAD51: implications for DNA repair and cancer. *DNA Repair (Amst.)*, **2**, 1015–1028.
20. Baumann,P., Benson,F.E., Hajibagheri,N. and West,S.C. (1997) Purification of human Rad51 protein by selective spermidine precipitation. *Mutat. Res.*, **384**, 65–72.
21. Henricksen,L.A., Umbricht,C.B. and Wold,M.S. (1994) Recombinant replication protein A: expression, complex formation, and functional characterization. *J. Biol. Chem.*, **269**, 11121–11132.
22. Gill,S.C. and von Hippel,P.H. (1990) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.*, **189**, 319–326.
23. Lobley,A. and Wallace,B.A. (2001) DICHROWEB: a website for the analysis of protein secondary structure from circular dichroism spectra. *Biophys. J.*, **80**, 373a.
24. Lobley,A., Whitmore,L. and Wallace,B.A. (2002) DICHROWEB: an interactive website for the analysis of protein secondary structure from circular dichroism spectra. *Bioinformatics*, **18**, 211–212.
25. Sigurdsson,S., Trujillo,K., Song,B., Stratton,S. and Sung,P. (2001) Basis for avid homologous DNA strand exchange by human Rad51 and RPA. *J. Biol. Chem.*, **12**, 8798–8806.
26. Sung,P. and Robberson,D.L. (1995) DNA strand exchange mediated by a RAD51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. *Cell*, **82**, 453–461.

27. Sung,P. (1994) Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. *Science*, **265**, 1241–1243.
28. Baumann,P., Benson,F.E. and West,S.C. (1996) Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions *in vitro*. *Cell*, **87**, 757–766.
29. Baumann,P. and West,S.C. (1997) The human Rad51 protein: polarity of strand transfer and stimulation by hRP-A. *EMBO J.*, **16**, 5198–5206.
30. Gupta,R.C., Bazemore,L.R., Golub,E.I. and Radding,C.M. (1997) Activities of human recombination protein Rad51. *Proc. Natl Acad. Sci. USA*, **94**, 463–468.
31. Baumann,P. and West,S.C. (1998) Role of the human RAD51 protein in homologous recombination and double-stranded-break repair. *Trends Biochem. Sci.*, **23**, 247–251.
32. Venkitaraman,A.R. (2000) The breast cancer susceptibility gene, BRCA2: at the crossroads between DNA replication and recombination? *Philos. Trans. R Soc. Lond B Biol. Sci.*, **355**, 191–198.
33. Bugreev,D.V. and Mazin,A.V. (2004) Ca^{2+} activates human homologous recombination protein Rad51 by modulating its ATPase activity. *Proc. Natl Acad. Sci. USA*, **101**, 9988–9993.
34. Liu,Y., Stasiak,A.Z., Masson,J.Y., McIlwraith,M.J., Stasiak,A. and West,S.C. (2004) Conformational changes modulate the activity of human RAD51 protein. *J. Mol. Biol.*, **337**, 817–827.
35. Bugreev,D.V., Golub,E.I., Stasiak,A.Z., Stasiak,A. and Mazin,A.V. (2005) Activation of human meiosis-specific recombinase Dmc1 by Ca^{2+}. *J. Biol. Chem.*, **280**, 26886–26895.
36. Schmitz,C., Perraud,A.L., Johnson,C.O., Inabe,K., Smith,M.K., Penner,R., Kurosaki,T., Fleig,A. and Scharenberg,A.M. (2003) Regulation of vertebrate cellular Mg^{2+} homeostasis by TRPM7. *Cell*, **114**, 191–200.
37. Romani,A.M. and Scarpa,A. (2000) Regulation of cellular magnesium. *Front. Biosci.*, **5**, D720–734.
38. Alberts,B.R.K., Lewis,J., Raff,M., Walter,P. and Johnson,J. (2000) *Molecular Biology of the Cell*. 4th edn. Garland.
39. Wu,Y., He,Y., Moya,I.A., Qian,X. and Luo,Y. (2004) Crystal structure of archaeal recombinase RadA: a snapshot of its extended conformation. *Mol. Cell*, **15**, 423–435.
40. San Filippo,J., Chi,P., Sehorn,M.G., Etchin,J., Krejci,L. and Sung,P. (2006) Recombination mediator and RAD51 targeting activities of a human BRCA2 polypeptide. *J. Biol. Chem.*, **281**, 11649–11657.
41. Brini,M., Murgia,M., Pasti,L., Picard,D., Pozzan,T. and Rizzuto,R. (1993) Nuclear Ca^{2+} concentration measured with specifically targeted recombinant aequorin. *EMBO J.*, **12**, 4813–4819.
42. Tutt,A. and Ashworth,A. (2002) The relationship between the roles of BRCA genes in DNA repair and cancer predisposition. *Trends Mol. Med.*, **8**, 571–576.
43. Yang,H., Li,Q., Fan,J., Holloman,W.K. and Pavletich,N.P. (2005) The BRCA2 homologue Brh2 nucleates RAD51 filament formation at a dsDNA–ssDNA junction. *Nature*, **433**, 653–657.
44. Chen,J., Silver,D.P., Walpita,D., Cantor,S.B., Gazdar,A.F., Tomlinson,G., Couch,F.J., Weber,B.L., Ashley,T., Livingston,D.M. *et al.* (1998) Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. *Mol. Cell*, **2**, 317–328.
45. Tsukuda,T., Fleming,A.B., Nickoloff,J.A. and Osley,M.A. (2005) Chromatin remodelling at a DNA double-strand break site in *Saccharomyces cerevisiae*. *Nature*, **438**, 379–383.
46. Shivji,M.K. and Venkitaraman,A.R. (2004) DNA recombination, chromosomal stability and carcinogenesis: insights into the role of BRCA2. *DNA Repair (Amst.)*, **3**, 835–843.